CARDIAC AND PULMONARY REPLACEMENT

PERINATAL INDUCTION OF IMMUNOTOLERANCE TO CARDIAC AND PULMONARY ALLOGRAFTS

David D. Yuh, MD
Kimberly L. Gandy, MD
Bruce A. Reitz, MD
Grant Hoyt
Robert C. Robbins, MD

Objectives: **Tolerance appears to be more easily induced in the fetus before full immunocompetence is established, but elucidation of this process is needed. A model of perinatal tolerance induction to neonatal skin allografts followed by cardiac and pulmonary allografts is described.** ***Methods:*** **Sixty Lewis ($RT1^l$) rat fetuses were inoculated intraperitoneally at 18 days' gestation with 1×10^7 ACI ($RT1^a$) rat fetal liver cells (group I); 20 Lewis fetuses were inoculated with 2×10^7 ACI fetal liver cells (group II). Control groups consisted of Lewis fetuses inoculated with saline solution ($n = 25$, group III) and fetuses that were not inoculated ($n = 25$, group IV). Twenty-five of the 50 surviving group I rats received ACI skin (<24 hours old) and heart (8 to 10 weeks old) allografts (group IA); the remaining 25 rats received only ACI heart grafts (group IB). Groups II, III, and IV received ACI skin and cardiac allografts. Recipients tolerant to both skin and cardiac grafts received orthotopic ACI lung grafts and third-party skin grafts. Tolerance was indicated by graft survival for more than 100 days. Limiting dilution and flow cytometric analyses were performed.** ***Results:*** **Abortion rates in groups I, II, III, and IV were 17% (10/60), 65% (13/20), 8% (2/25), and 4% (1/25), respectively. Specific tolerance to skin, cardiac, and lung allografts was observed in seven of 25 group IA recipients (28%) and seven of seven group II recipients (100%) compared with no tolerance in any group IB, III, or IV recipients ($p = 0.03$, χ^2 test). A 100-fold reduction of precursor cytotoxic T lymphocytes and significant splenocyte and bone marrow chimerism in tolerant versus nontolerant rats were noted ($p = 0.0001$, Student's t test).** ***Conclusions:*** **Using donor-strain fetal liver cells and neonatal skin grafts, we achieved higher frequencies of tolerance to solid organ grafts in adulthood with lower cell inocula and abortion rates than previously described. Chimerism and depressed precursor cytotoxic T lymphocyte frequencies in tolerant recipients suggest that hematopoietic stem cell engraftment and clonal deletion/anergy are involved in induction of perinatal tolerance. (J Thorac Cardiovasc Surg 1997;114:64-75)**

From the Department of Cardiothoracic Surgery, Stanford University Medical Center, Stanford, Calif.

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Address for reprints: David D. Yuh, MD, Department of Surgery, Room H3680, Stanford University Medical Center, 300 Pasteur Dr., Stanford, CA 94305.

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Congenital defects of the heart and great vessels can be detected in utero as early as the second trimester of gestation by means of echocardiographic imaging.[1] Some of these defects are not amenable to conventional postnatal surgical correction (e.g., some variants of the hypoplastic left heart syndrome and pulmonary atresia with intact ventricular septum), are associated with high early mortality rates, and may require cardiac transplantation in the neonatal and pediatric periods for any realistic chance for long-term survival. Unfortunately, a significant number of neonates will die awaiting cardiac transplantation or shortly after transplantation.

Moreover, the side effects of conventional immunosuppression are particularly extreme in the neonatal and infant populations. Consequently, research directed toward developing a means to confer immunologic tolerance during fetal development in anticipation of cardiac transplantation has garnered interest in recent years.

Experimental evidence that fetuses are tolerant of allogeneic tissue grafts is abundant. In 1953, Billingham, Brent, and Medawar[2] described the active induction of immunologic tolerance in mice by exposing the recipient fetus to alloantigen. Recent studies in ovine and nonhuman primate models have clearly demonstrated that the early gestational fetus is tolerant of alloantigens and is an optimal recipient of allogeneic hematopoietic stem cells (HSCs). HSCs are the pluripotent progenitor cells from which key immune cell lines are derived (e.g., lymphocytes, monocytes, granulocytes). Zanjani,[3] Harrison,[4] and their colleagues have successfully introduced allogeneic HSCs into fetal sheep and rhesus monkeys with subsequent multilineage engraftment and without evidence of graft-versus-host disease (GVHD).[3, 4] However, despite the successful in utero induction of low levels of hematopoietic chimerism in sheep, tolerance to subsequent renal allografts was not achieved initially.[5] Further technical modifications have recently led to in utero tolerance induction to postnatal renal allografts.[6] Nevertheless, the relatively undefined immunologic makeup and high cost of these large animal models have precluded incisive mechanistic studies of HSC engraftment and active tolerance induction in utero. To this end, we have developed a relatively inexpensive, reproducible, and well-characterized rodent model of perinatal tolerance induction to skin, cardiac, and lung allografts using donor-strain fetal liver cells as a source of HSCs.

Methods

Animals. Adult timed-pregnant female Lewis (LEW and $RT1^l$, 8 to 10 weeks old) and ACI (ACI and $RT1^a$, 8 to 10 weeks old) rats at 17 days' gestation and adult male ACI, LEW, and Brown-Norway (BN, $RT1^b$, 8 to 10 weeks old) rats were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. All animals were maintained in the animal care facilities of the Department of Cardiothoracic Surgery, Stanford University Medical Center, Stanford, California. Their environment was maintained at 21° ± 2° C with a time-regulated light period from 7:00 AM to 7:00 PM. Microisolator-system cages housed three rats per cage except in the case of the timed-pregnant rats, which were housed individually. Rats were provided water and dry food ad libitum. Periodic serologic analysis of room sentinel animals showed that all rats were free of acute viral infection. All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Fetal liver cell preparation. After being anesthetized with sodium pentobarbital (25 mg/kg intraperitoneally) and supplemental methoxyflurane (inhalational), timed-pregnant ACI rats at 18 days' gestation underwent a midline laparotomy with delivery of the bicornuted uterus. Each limb of the uterus was incised along its longitudinal axis and the fetuses were separated from their placental attachments. The fetuses underwent laparotomy with extrusion and excision of the livers en bloc. The livers were immediately placed into phosphate-buffered saline solution (PBS) at 4° C and homogenized. The homogenate was layered on a Lympholyte-Rat gradient (Accurate Chemical & Scientific Corp., Westbury, N.Y.) and centrifuged (1000*g* at 20° to 25° C for 20 minutes). After the supernatant layer had been discarded, the interface layer containing nucleated fetal liver cells was aspirated and placed in a 50 ml conical centrifuge tube and washed three times in PBS (400*g* at 4° C for 10 minutes). The fetal liver cell suspension concentration was adjusted to obtain 1×10^7 cells/20 μl PBS.

In utero fetal inoculation. With methoxyflurane anesthesia, each timed-pregnant LEW rat at 18 days' gestation underwent a midline laparotomy with delivery of the bicornuted uterus. Under direct vision aided by transillumination, each of 60 LEW fetuses (five litters) were inoculated intraperitoneally with 1×10^7 ACI fetal liver cells (group I) with the use of a Hamilton repeating syringe (Hamilton Corp., Reno, N.V.) fitted with a 30-gauge needle. Care was taken to avoid tearing the fetal tissues with the needle or avulsing the uterine vessels. Twenty LEW fetuses (three litters) were similarly inoculated with 2×10^7 ACI fetal liver cells (group II). For controls, each of 25 LEW fetuses (three litters) were inoculated with 20 μl of PBS alone (group III), and another 25 LEW fetuses (two litters) received no inoculations (group IV).

After the inoculations were completed, the laparotomy incisions were closed in two layers with running 4-0 Vicryl sutures (Ethicon, Inc., Somerville, N.J.). After recovering from anesthesia, the pregnant rats were returned to their respective cages and allowed to come to term.

ACI to LEW neonatal skin transplantation. With the aid of deep hypothermia, 4 × 4 mm full-thickness sections of neonatal ACI skin were grafted onto 25 of the 50 group I LEW neonates that survived to term; this subgroup was designated group IA. Group IB consisted of the remaining group I neonates, which did not receive skin grafts. All of the surviving group II ($n = 7$), III ($n = 23$), and IV ($n = 24$) LEW neonates received neonatal ACI skin grafts. After receiving the grafts, the neonates were slowly rewarmed to 37° C, returned to their respective maternal rats, and permitted to grow and develop normally over the next 8 to 10 weeks. No neonates died after skin grafting.

Table I. *Abortion rates and ACI allograft tolerance rates among Lewis rats receiving in utero inoculation with ACI fetal liver cells, PBS, and/or no inoculation*

Group (in utero inoculum)	*No. of fetuses*	*No. of neonates*	*Abortion rate (%)*	*Tolerance rate (%)**
Group I (1×10^7 fetal cells)	60	50	17†	28†
Group IA—with skin graft	—	25	—	28†
Group IB—without skin graft	—	25	—	0
Group II (2×10^7 fetal cells)	20	7	65	100†
Group III (PBS)	25	23	8	0
Group IV (nothing)	25	24	4	0

*Tolerance to ACI neonatal skin and/or adult cardiac/lung allografts defined as graft survival more than 100 days.
†Rates significantly greater compared with corresponding values in the other groups ($p = 0.03$; χ^2 analysis).

ACI to LEW heterotopic cardiac transplantation. With the animals under sodium pentobarbital (50 mg/kg intraperitoneal) anesthesia, the hearts from adult ACI donor rats were transplanted into all mature group IA, IB, II, III, and IV LEW rats (8 to 10 weeks old) by means of the technique described by Ono and Lindsey.[7]

ACI to LEW orthotopic lung transplantation. With the aid of sodium pentobarbital (50 mg/kg intraperitoneal) and enflurane (1 to 2 volume percent) endotracheal anesthesia, left lungs from adult ACI donor rats were transplanted orthotopically into all recipients deemed tolerant to skin and cardiac allografts (group IA, $n = 7$; group II, $n = 7$) by means of a modification of the technique described by Marck and Wildevuur.[8]

BN to LEW third-party adult skin grafts. With sodium pentobarbital (50 mg/kg intraperitoneal) anesthesia, 2×2 cm sections of full-thickness skin, procured from adult male BN rats, were grafted onto the flanks of five naive LEW adult male rats (controls) and all ACI-tolerant group IA and group II LEW recipients ($n = 14$) 2 weeks after lung transplantation.

Skin, cardiac, and lung allograft evaluation. Neonatal ACI skin allografts were evaluated by daily visual inspection. Viability was indicated by pigmented (brown) hair growth; rejection was indicated by graft sloughing. ACI cardiac allografts were assessed by daily abdominal palpation. Viability was evidenced by strong graft contractions; rejection was indicated by the complete absence of ventricular contractions. Graft rejection or tolerance was confirmed with hematoxylin and eosin histologic analyses of myocardial biopsy specimens taken at the time of rejection or after 100 days, respectively. Pulmonary grafts were graded according to the degree of radiographic opacification. Radiographs were subjected to a five-point grading scale from 0 to 4 (0 = complete opacification, 4 = completely clear lung field). Changes in grades were confirmed and correlated with hematoxylin and eosin histologic analyses of open lung biopsy specimens. Graft rejection was defined as complete radiographic opacification coupled with mononuclear cell infiltration, hemorrhagic vasculitis, or tissue necrosis. Tolerance for all grafts was defined as survival in excess of 100 days after transplantation. Rats in which graft rejection occurred were designated "nontolerant."

Flow cytometric analysis of recipient peripheral blood lymphocytes, splenocytes, and bone marrow. Peripheral blood lymphocytes (PBLs), splenocytes, and bone marrow obtained from all tolerant (after heart and lung transplantation) and randomly selected nontolerant adult LEW recipients (12 to 14 weeks) in groups IA ($n = 12$; 7 tolerant, 5 nontolerant), IB ($n = 5$), II ($n = 7$), III ($n = 5$), and IV ($n = 5$) were analyzed with flow cytometry (FACScan, Becton Dickinson and Company, Rutherford, N.J.) to measure the presence of allogeneic (ACI) chimerism. Naive age-matched LEW ($n = 5$) and ACI ($n = 5$) rats were used as negative and positive controls, respectively.

First, approximately 200 μl of peripheral blood was collected in 10 μl ethylenediaminetetraacetic acid by means of tail vein puncture from all rats. Second, small portions (25%) of splenic tissue were obtained from these rats through a 2 cm left subcostal incision performed with the rats receiving sodium pentobarbital anesthesia. Finally, femoral bone marrow was aspirated from each rat. These tissues were collected and homogenized into a single-cell suspension in PBS at 4° C. For each blood, splenocyte, and bone marrow sample, most red cells were eliminated in the pellet after sedimentation on a dextran gradient. The residual red cells were lysed with ammonium chloride. Fc receptors were blocked with rat immunoglobulin G or normal rat serum. Cells were stained on ice for donor haplotype with fluorescein isothiocyanate–conjugated mouse antirat $RT1^a$ monoclonal antibodies (PharMingen Research Products, San Diego, Calif.). Cells were washed in PBS with a fetal calf serum cushion and stained with propidium iodide to exclude dead cells. Flow cytometry was used to determine the percentage of donor haplotype cells in each animal. Dead cells and debris were gated out with forward scatter, orthogonal scatter, and propidium iodide staining profiles. Results were expressed as the percentage of cells expressing the donor marker ($RT1^a$).

Limiting dilution assay for cytotoxic lymphocyte precursors. Splenocyte samples were taken from the same rats that underwent flow cytometric analysis and were assayed for precursor cytotoxic T lymphocyte (CTL) frequency; samples from within each group were pooled. LEW responder cells were plated from 20,000 to 1.28×10^6 cells per well (24 replicates per concentration) into round-bottom microtiter wells in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), 2 mmol/L L-glutamine, 5×10^{-5} mol/L β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 20% supernatant from concanavalin A–activated rat

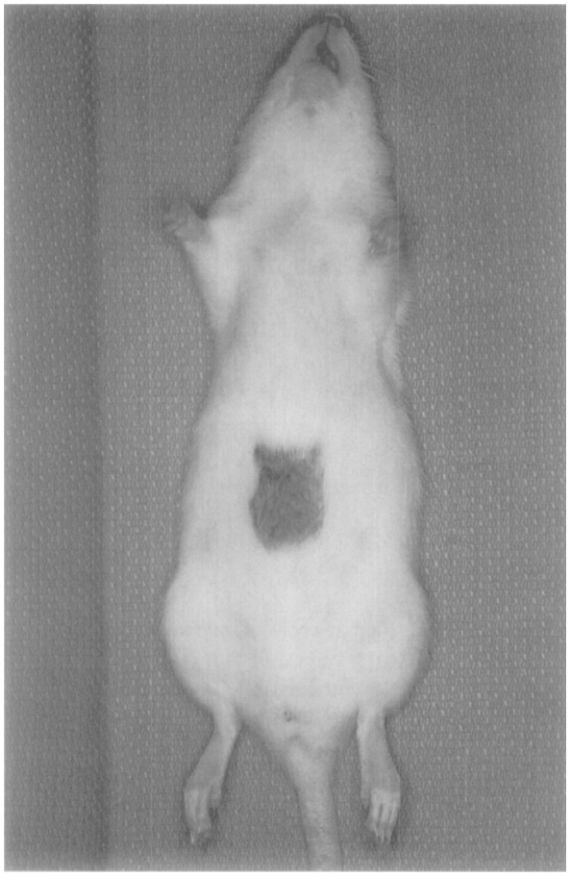

Fig. 1. Tolerance to donor-strain (ACI) skin graft. Photograph displays an ACI skin graft on a tolerant Lewis recipient after 8 postoperative weeks. Note the pigmented phenotype of the hair produced from the viable ACI skin graft.

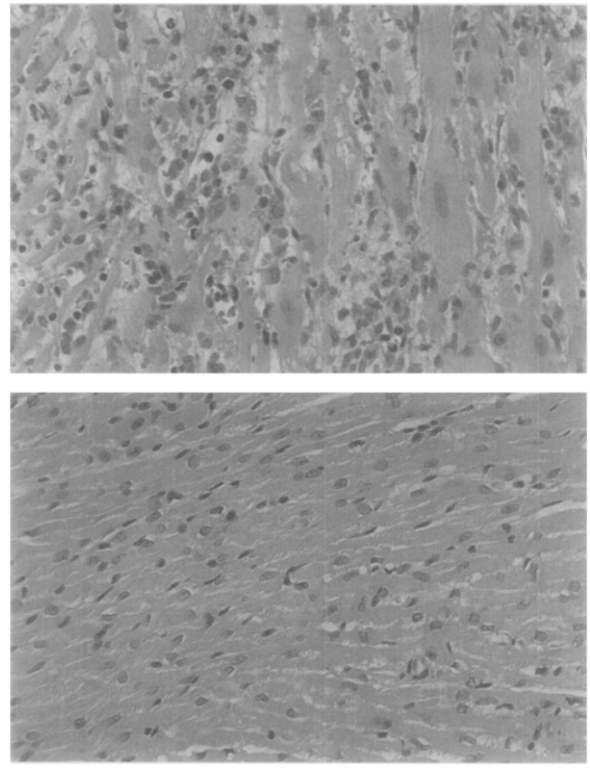

Fig. 2. Histologic analysis of ACI cardiac allografts in nontolerant and tolerant Lewis recipients. Note the extensive perivascular and interstitial mononuclear cell infiltrates in an ACI cardiac allograft rejected by a Lewis recipient on postoperative day 7 *(top)*. No histologic evidence of rejection was detected in ACI grafts sampled for biopsy 150 days after transplantation in tolerant Lewis rats *(bottom)*. (Hematoxylin and eosin stain; original magnification ×100.)

splenocytes, and 50 μmol/L α-methyl mannoside. Subsequently, 5×10^5 irradiated (2000 rads) ACI stimulator cells were added to each well. The plates were incubated in a humidified carbon dioxide incubator. After 5 days, aliquots from each well were tested for lysis of ^{51}Cr-labeled concanavalin A–activated ACI blasts. Aliquots of supernatant were counted in a gamma counter. Wells were considered positive if the specific release was greater than 10%.

Statistical analysis. Tolerance rates observed among the different groups were compared by means of χ^2 analysis. Graft survival times between groups were compared by means of the Mann-Whitney U test. Chimeric levels among the different groups were compared by means of the two-tailed Student's *t* test. Precursor CTL frequency was determined by linear regression.

Results

Fetal survival/abortions. Abortion rates among groups I, II, III, and IV were 17% (10/60), 65% (13/20), 8% (2/25), and 4% (1/25), respectively (Table I). The surviving progeny displayed no gross anatomic or physiologic abnormalities, including evidence of GVHD (e.g., hair loss, failure to thrive, diarrhea, or skin lesions), during their maturation.

Neonatal ACI to LEW skin allograft survival. Twenty-eight percent (7/25) of group IA and 100% (7/7) of group II LEW recipients of ACI neonatal skin allografts clearly displayed pigmented hair growth characteristic of the ACI phenotype by postoperative day 28, indicating graft viability (Fig. 1). These allografts have remained viable for more than 250 days without evidence of rejection. In contrast, all of the ACI neonatal skin allografts on the remaining nontolerant group IA (18/25; 72%), group III (23/23; 100%), and group IV (24/24; 100%) recipients were uniformly sloughed off by postoperative day 7, indicating acute allograft rejection.

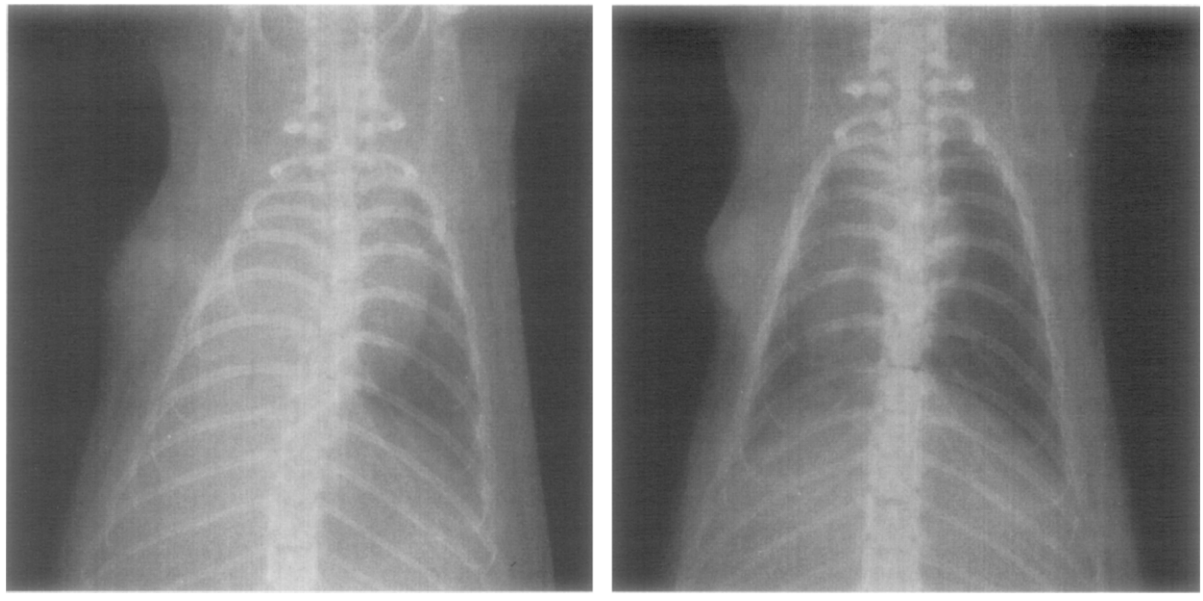

Fig. 3. Chest radiographs of nontolerant *(left)* and tolerant *(right)* lung allograft recipients. The left lung field (reversed projection) is opacified in the nontolerant recipient, indicating rejection by postoperative day 7, compared with the nearly clear corresponding field in the tolerant animal 100 days after transplantation.

Adult ACI to LEW cardiac allograft survival. All group IA and II recipients demonstrating tolerance to ACI skin allografts likewise achieved ACI cardiac allograft survival times in excess of 200 days to date (group IA, $n = 7$; group II, $n = 7$), evidenced by continued strong graft contractions. Pathologic analysis of these grafts revealed no gross or histologic evidence of rejection (Fig. 2). In the remaining nontolerant group IA ($n = 18$), group IB ($n = 25$), group III ($n = 23$), and group IV ($n = 24$) recipients, cardiac allograft rejection occurred at 7.0 ± 0.3, 6.9 ± 0.4, 7.1 ± 0.5, and 6.9 ± 0.5 days ($p = 0.35$), respectively.

Adult ACI to LEW pulmonary allograft survival. All group IA and II recipients demonstrating tolerance to ACI skin and cardiac allografts likewise have ACI lung allograft survival times in excess of 100 days to date (group IA, $n = 7$; group II, $n = 7$). Allograft tolerance was indicated with serial radiographs (Fig. 3) and confirmed with pathologic analysis of graft tissue (Fig. 4).

Adult BN to LEW third-party skin allograft survival. Third-party Brown-Norway skin graft survival times for control ($n = 5$), tolerant group IA ($n = 7$), and group II (= 7) recipients were 7.0 ± 1.0, 7.0 ± 0.8, and 7.0 ± 0.0 days, respectively, and were not significantly different among the groups ($p = 1.00$).

Flow cytometric analysis for PBL, splenocyte, and bone marrow allogeneic chimerism. Analysis of the PBL populations among all randomly selected animals revealed no significant peripheral blood chimerism. However, significant chimerism was detected in splenocytes (Table II, Fig. 5) from all tolerant group IA (1.5% ± 0.05%, $p = 0.0001$) and group II recipients (1.7% ± 0.07%, $p = 0.0001$). Generally, higher degrees of chimerism were noted in bone marrow populations (Table II, Fig. 6) from group IA (55.1% ± 25.2%, $p = 0.0001$) and group II (60.4% ± 23.6%, $p = 0.0001$). No splenocyte or bone marrow chimerism was detected in the nontolerant group IA or group IB recipients, or in the group III and IV controls.

Limiting dilution assay for CTL precursors. Limiting dilution analysis revealed a fivefold reduction of precursor CTLs in tolerant group IA and II graft recipients compared with naive LEW controls ($p = 0.0001$) and a 100-fold reduction compared with the nontolerant recipients in groups IA, IB, III, and IV ($p = 0.0001$) (Table III).

Discussion

Patients with complex congenital cardiac defects that are not amenable to conventional postnatal surgical correction are often referred for transplan-

tation. Unfortunately, in the neonatal and pediatric populations, cardiac transplantation is severely limited by organ availability, acute and chronic graft rejection, and long-term pharmacologic immunosuppression with its attendant side effects.[9] Noninvasive prenatal diagnoses of anomalies of the heart and great vessels has provided cardiologists and cardiac transplant surgeons with a window of opportunity during which allogeneic and, possibly, xenogeneic tolerance can be induced during critical developmental stages of the immune system. This window is indeed narrow, because the human fetal immune system becomes competent around the fifteenth or sixteenth gestational week and fetal safety precludes injections before the thirteenth week.

Reported successes in the treatment of a broad spectrum of genetic disorders with postnatal bone marrow transplantation has sparked interest in the use of pluripotent HSCs to induce allogeneic and xenogeneic tolerance in utero.[10] The fetus is an ideal host for allogeneic HSC transplantation because of its ontogenetic readiness for engraftment and its immunodeficient state before the sixteenth gestational week. During fetal development, the expansion of the fetal bone marrow compartment and HSC distribution patterns may contribute to the receptivity of the marrow environment to foreign stem cells. In human beings, HSCs are first noted in the yolk sac during the fourth week of gestation.[11] From there, the liver and spleen are seeded during the sixth and seventh weeks, respectively. The bone marrow is, in turn, seeded by the liver and assumes hematopoietic function by the twentieth week. Therefore it follows that the optimal time to inoculate the fetus with donor-derived HSCs might be between the thirteenth and sixteenth weeks, before immunocompetence is established and before the bone marrow is seeded with native HSCs from the liver. Furthermore, it has been postulated that donor HSCs might better compete with native HSCs for hematopoietic niches in the marrow if they were introduced continuously or repeatedly as opposed to a single large dose. A well-defined rodent model of in utero allogeneic stem cell engraftment would facilitate investigation of these issues.

Since the initial work reported by Billingham, Brent, and Medawar[2] in 1953, several rodent models of tolerance induction have been designed. Unfortunately, recent attempts at inducing tolerance in utero using rodent models of transplantation have met with difficulty in achieving adequate tolerance

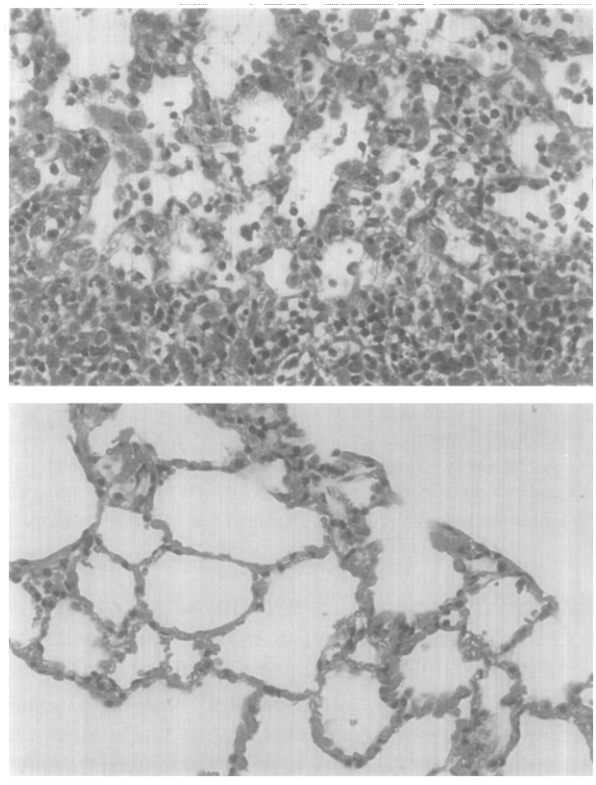

Fig. 4. Histologic analysis of ACI lung allografts in nontolerant and tolerant Lewis recipients. Note the extensive perivascular mononuclear cell infiltrates extending into the adjacent alveolar septa in an ACI lung allograft rejected by a Lewis recipient on postoperative day 7 *(top)*. No histologic evidence of rejection was detected in ACI grafts sampled for biopsy 100 days after transplantation in tolerant Lewis rats *(bottom)*. (Hematoxylin and eosin stain; original magnification ×100.)

rates for further study. Chen and McCullagh[12] found that although bone marrow cells administered in utero induced tolerance in certain strain combinations, spleen cells syngeneic with demonstrably tolerogenic bone marrow cells not only invariably failed to induce tolerance, but commonly resulted in sensitized recipients. The inability of the inoculated splenocytes to migrate into the fetal circulation from the peritoneal space was excluded as a barrier to tolerance induction, because the cells were administered intravenously. Kline and colleagues[13] were successful in achieving tolerance in a semiallogeneic rat model of cardiac transplantation by inoculating fetal recipients with adult donor-strain splenocytes; however, a high cell inoculum (5×10^7 cells/fetus) resulting in a high abortion rate (94%) was required to induce tolerance. In light of these difficulties, we

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Table II. *Flow cytometric analyses of adult recipient splenocyte and bone marrow populations*

*Group**	*n*	*Splenocyte ACI ($RT1^a$) phenotype (mean % ± SD)*	*Bone marrow ACI ($RT1^a$) phenotype (mean % ± SD)*	*p Value†*
ACI positive control (no graft)	5	99.4 ± 0.05	99.3 ± 0.03	—
Lewis negative control (no graft)	5	0.2 ± 0.03	0.1 ± 0.04	—
Group IA (**tolerant**)	7	1.5 ± 0.05	55.1 ± 25.2	**0.0001**
Group IA (nontolerant)	5	0.2 ± 0.05	0.3 ± 0.03	1.0000
Group IB (no skin graft)	5	0.2 ± 0.05	0.2 ± 0.04	1.0000
Group II (**tolerant**)	7	1.7 ± 0.07	60.4 ± 23.6	**0.0001**
Group III (PBS inoculum in utero)	5	0.3 ± 0.08	0.2 ± 0.03	1.0000
Group IV (no inoculum in utero)	5	0.1 ± 0.02	0.2 ± 0.01	1.0000

*Group IA—in utero inoculation with 1×10^7 ACI fetal liver cells; neonatal skin and adult cardiac/lung allografts.
Group IB—in utero inoculation with 1×10^7 ACI fetal liver cells; adult cardiac allograft only.
Group II—in utero inoculation with 2×10^7 ACI fetal liver cells; neonatal skin and adult cardiac/lung allografts.
Group III—in utero inoculation with PBS; neonatal skin and adult cardiac allografts.
Group IV—no in utero inoculation; neonatal skin and adult cardiac allografts.
†*p* Values derived from two-tailed Student's *t* test; all comparisons made with Lewis negative controls.

developed a more efficient perinatal tolerizing protocol using HSCs instead of mature splenocytes.

In these studies, we demonstrate that tolerance to neonatal skin and adult cardiac and lung tissue across a strong allogeneic mismatch can be initiated by inoculating the recipient fetuses with donor-strain fetal liver cells. T cell–depleted fetal liver tissue and umbilical cord blood are believed to be particularly enriched in HSCs and are tolerogenic elements well-suited for in utero administration. These elements are relatively easy to obtain and do not appear to produce GVHD in the recipient, presumably because of a lack of mature competent T lymphocytes. By using fetal liver cells instead of adult splenocytes as the tolerogen, we achieved a tolerant state with markedly fewer cells than described by Kline and colleagues.[13] The "immortality" of HSCs, not observed with adult splenocytes, may be critical for the maintenance of tolerance. In fairness, we cannot be certain that the inductive elements of the fetal liver inoculum used in our model are indeed HSCs, because specific rat HSC markers have not yet been identified. However, on the basis of experience with fetal liver cells in the context of other animal models,[4, 14] this appears likely. Clinical application of this concept might involve the intrauterine administration of fetal liver cells or T cell–depleted cord blood to the afflicted fetus as opposed to purified HSCs. In fact, HSC engraftment may be augmented by a separate "facilitator" cell subpopulation[15] that is present in whole bone marrow and, quite possibly, fetal liver tissue. These facilitator cells might be excluded in the process of obtaining a purified HSC inoculum.

Although we achieved a higher frequency of tolerance induction in our model compared with other studies, the failure to induce tolerance in 18 of 25 identically treated rats warrants discussion. It is generally believed that HSC engraftment in utero is enhanced if such cells are introduced in the early stages of gestation and hematolymphoid development; however, the optimal time of inoculation has not been defined. It is possible that HSC engraftment did not occur in nontolerant recipients because they were immunocompetent at 18 days' gestation. Technical failure in delivering the fetal liver cells into the fetal peritoneal space may have also accounted for engraftment failure, although preliminary trials with dye indicated that our method of injection was adequate. Finally, we noted that increasing the dose of fetal liver cells increases the frequency of tolerance induction, albeit at the expense of higher abortion rates. Performing serial injections at lower cell doses throughout gestation may improve tolerance induction rates while preserving low rates of abortion.

Although significantly lower abortion rates were observed with this model compared with rates observed with other rodent models of in utero tolerance induction, we can only speculate as to the precise cause of the abortions. We noted no evidence of GVHD in any surviving animals. Nevertheless, GVHD mediated by mature T cells in the fetal liver inoculum may have caused the abortions, although lymphoid cells isolated from recipient fetal livers were nonreactive against donor target cells in mixed lymphocyte culture (data not shown). Interestingly, abortions among similarly inoculated fe-

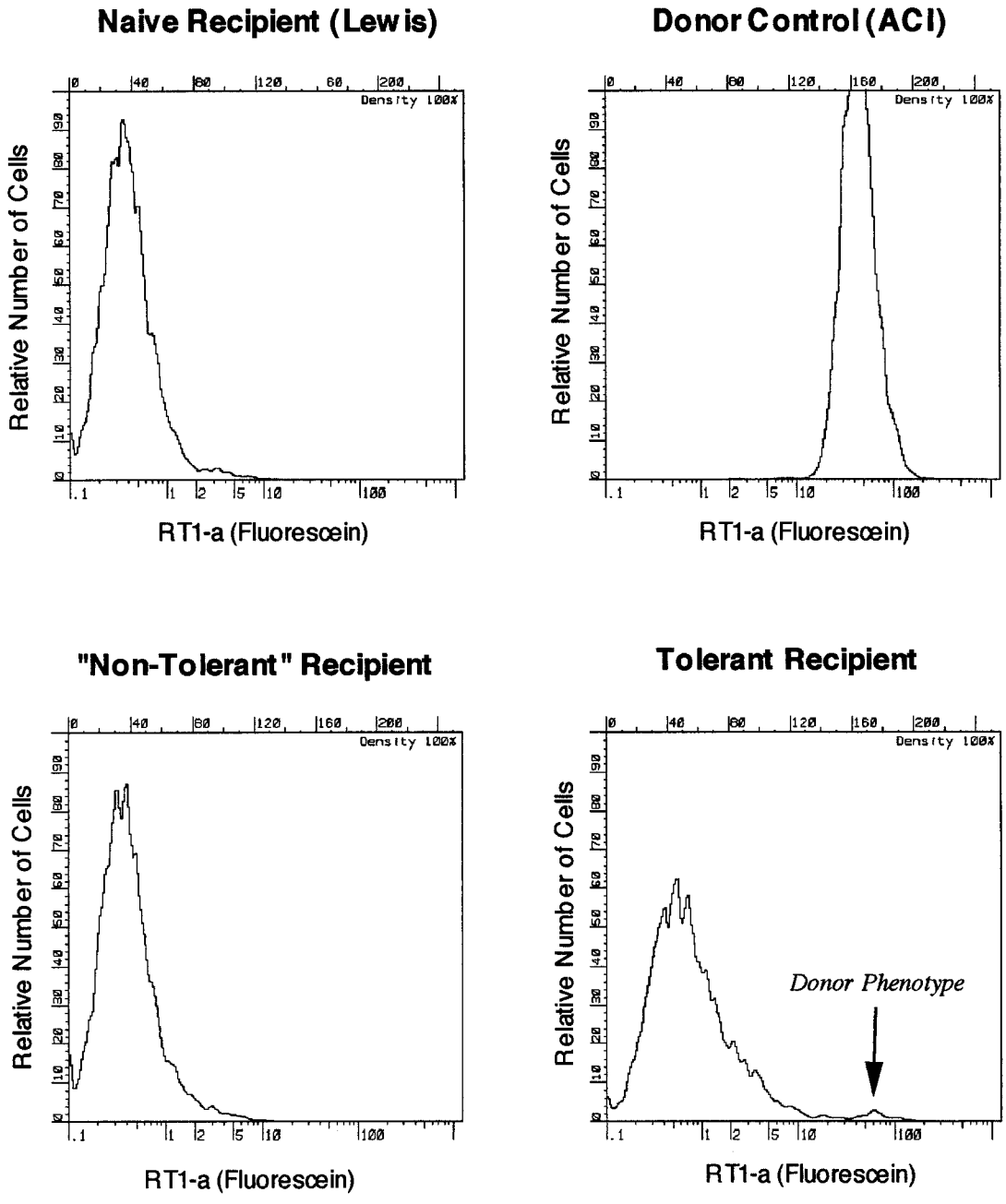

Fig. 5. Representative flow cytometric plots of splenocyte populations obtained from an adult naive recipient (Lewis), donor-strain positive control (ACI), and Lewis recipients inoculated with ACI fetal liver cells in utero (tolerant and nontolerant). Note the small subset of splenocytes expressing the donor phenotype in the tolerant rat.

tuses appeared to follow an "all-or-none" pattern; entire litters either aborted or were wholly preserved, which suggests that the intrauterine death of a few or even a single fetus might be detrimental to the entire litter. Mechanical trauma to the fetal tissues during inoculation appears to be excluded as a significant cause, because a very low abortion rate was noted among fetuses inoculated with PBS alone. It was not technically feasible to achieve perfectly aseptic conditions throughout the cell preparation

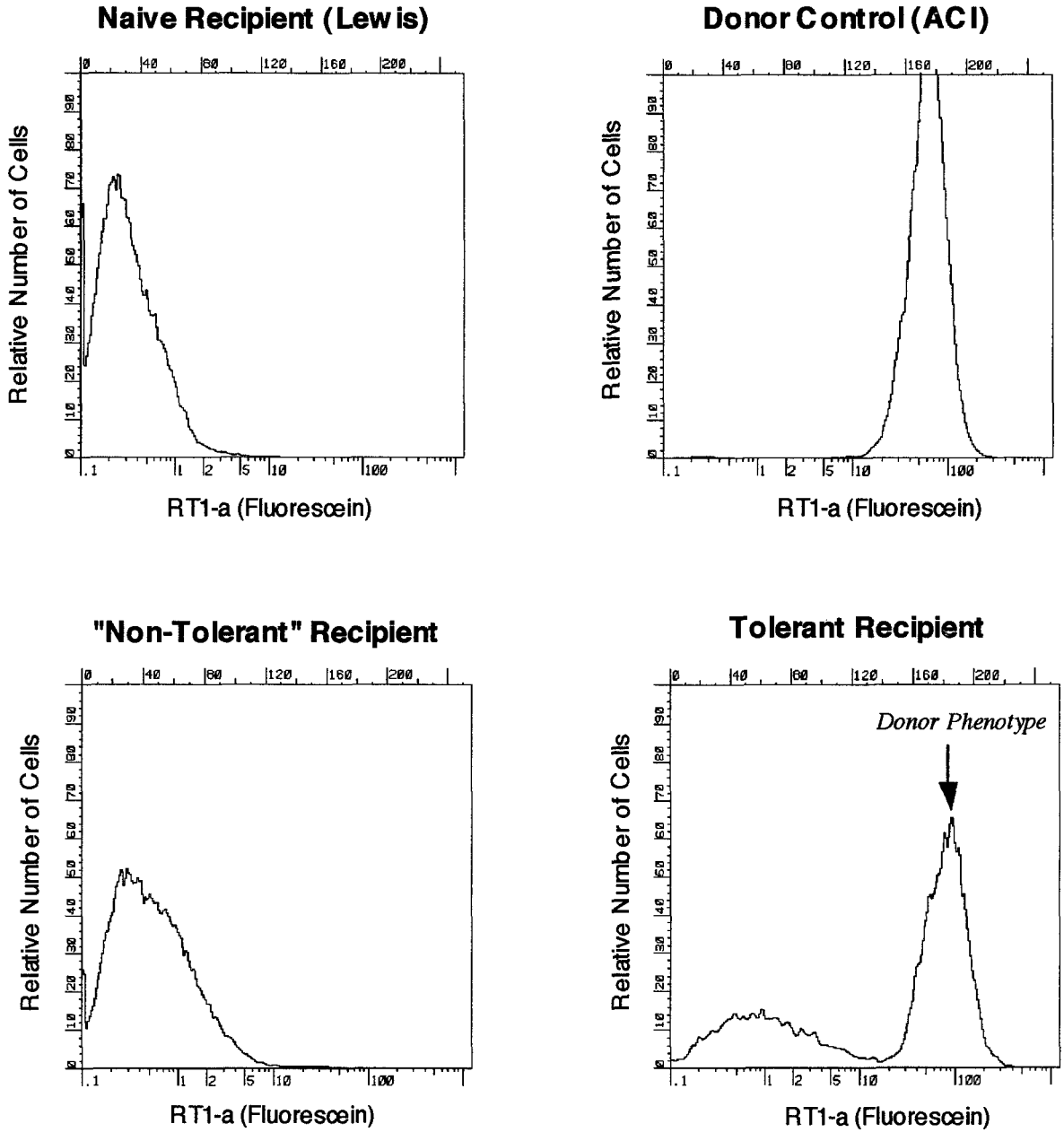

Fig. 6. Representative flow cytometric plots of bone marrow populations obtained from an adult naive recipient (Lewis), donor-strain positive control (ACI), and Lewis recipients inoculated with ACI fetal liver cells in utero (tolerant and nontolerant). Note the large subset of marrow cells expressing the donor phenotype in the tolerant rat.

and inoculating procedures. Thus it is possible that fetal death resulted from infection. Alternatively, the cellular component of the inoculum might be the contributing factor, because the abortion rates observed by our group and others appear to be proportional to the cell numbers used (data not shown). In our studies, doubling the cell inoculum from 10 million to 20 million cells resulted in a fourfold rise in abortions. Whether this phenomenon is a consequence of a graft-versus-host effect imparted by competent lymphocytes or, perhaps, of metabolic perturba-

Table III. *CTL precursor frequencies among adult recipient splenocyte populations*

*Group description**	*n*	*Precursor CTL frequency†*	*p Value‡*
Naive Lewis control (no graft)	5	1,650,000	—
Group IA (**tolerant**)	7	5,700,000	**0.0001‡**
Group IA (nontolerant)	5	55,425	0.0001
Group IB (no skin graft)	5	52,050	0.0001
Group II (**tolerant**)	7	5,500,000	**0.0001‡**
Group III (PBS inoculum in utero)	5	70,400	0.0001
Group IV (no inoculum in utero)	5	65,550	0.0001

*Group IA—in utero inoculation with 1×10^7 ACI fetal liver cells; neonatal skin and adult cardiac/lung allografts.
Group IB—in utero inoculation with 1×10^7 ACI fetal liver cells; adult cardiac allograft only.
Group II—in utero inoculation with 2×10^7 ACI fetal liver cells; neonatal skin and adult cardiac/lung allografts.
Group III—in utero inoculation with PBS; neonatal skin and adult cardiac allografts.
Group IV—no in utero inoculation; neonatal skin and adult cardiac allografts.

†CTL frequencies obtained by linear regression analysis.

‡Statistical comparisons made between each group versus control; comparisons also made between tolerant group IA and II recipients versus groups IA (nontolerant), IB, III, and IV.

tions caused by cell lysis products is presently unclear and deserving of further investigation.

Interestingly, we did not appreciate a uniformly significant level of hematopoietic chimerism among PBLs from the tolerant rats in early adulthood. Peripheral blood chimerism may, in fact, exist in these tolerant rats, but at a level that is undetectable by our assay. Relatively low but significant levels of chimerism were noted among the splenocyte populations. However, significantly higher proportions of bone marrow cells expressed the donor phenotype. Much has been written about the apparent correlation between the presence of hematopoietic chimerism and tolerance, but few studies have addressed the mechanisms behind this. One would be hard pressed to logically explain how a small subset of chimeric cells would be capable of preventing the majority of native lymphoid cells from delivering a swift, overwhelming assault against an allogeneic solid organ graft. A more palatable hypothesis might propose that chimerism is merely a by-product of, rather than the driving force behind, tolerance induction. In other words, chimerism may simply result when donor-strain HSCs, introduced into the recipient by direct inoculation or the transplanted organ itself, are merely permitted to survive and proliferate by a recipient immune system rendered tolerant by a mechanism that is not based on active immunoregulation effected by the HSCs. Therefore future studies should investigate a more passive role taken by HSCs in tolerance induction. For instance, it is widely established that thymic inoculation with alloantigen confers tolerance in several models of transplantation.[16-18] The introduction and subsequent engraftment of HSCs in utero would provide a self-perpetuating source of alloantigen that might directly influence the thymic education process. The durable presence of alloantigen may prove to be a key feature of future effective tolerance induction regimens.

We found that, in this model, allogeneic tolerance in adult life could be achieved only if donor-strain skin was grafted onto the recipient during the neonatal period after receiving donor-strain fetal liver cells in utero. Furthermore, unlike West, Morris, and Wood,[14] we found that the tolerance we induced was indeed donor-specific, because third-party Brown-Norway adult skin grafts were uniformly rejected among all recipients that were otherwise tolerant to ACI tissue. These findings, coupled with the significantly reduced CTL precursor frequencies observed in the tolerant recipients versus naive animals and nontolerant sensitized recipients, suggest that newborn recipients with newly engrafted allogeneic HSCs require early continuous exposure to corresponding antigenic determinants during a period of "education" to establish allogeneic tolerance. Several investigations have focused on the possible mechanisms of this phenomenon and suggested that exposure of the developing fetal immune system to donor antigenic determinants leads to a state of donor-specific unresponsiveness by clonal deletion or anergy. Kappler, Roehm, and Marrack[19] demonstrated that clonal deletion occurs in utero during the intrathymic development of T lymphocytes. Streilein[20] has described clonal anergy whereby reactive T cell clones become unresponsive when presented with the appropriate stimulus. Finally, we have not ruled out the induction of a suppressor cell population in these studies.

Although the prenatal induction of allogeneic

tolerance using T cell–depleted bone marrow from the solid organ donor might be useful for subsequent pulmonary, renal, or hepatic transplantation (e.g., living-related donors), clinical application of in utero tolerance induction for cardiac transplantation would most likely be realized in a xenogeneic context. Successful xenogeneic transplantation of human HSCs was initially reported in severe combined immunodeficient and lethally irradiated normal mice.[21, 22] Human-to-sheep and pig-to-monkey models of xenogeneic chimerism induced from in utero transplantation of HSCs have been described by Zanjani,[23] Tanaka,[24] and others. However, tolerance to solid organ xenografts has not yet been achieved. A basic understanding of the development of immunocompetence in utero and the role of chimerism in tolerance is needed to establish a coherent strategy for perinatal tolerance induction.

Tolerance induction in the neonatal as opposed to the prenatal period would certainly be more conducive to clinical allogeneic cardiac transplantation. West, Morris, and Wood[14] recently reported the use of fetal liver cells to induce tolerance to cardiac allografts in neonatal mice. Interestingly, they found that the induced tolerance was not donor-specific; prolongation of third-party allografts, as well as donor-type grafts, was observed. This finding enhances the clinical potential of this modality in that unmatched fetal liver HSCs might be used. Nevertheless, we do not believe that it is correct to assume, as some investigators have, that the neonatal and fetal immune systems are functionally similar. We clearly demonstrate that untreated neonatal rats uniformly and vigorously reject neonatal skin allografts despite the grafts being applied within 24 hours of birth, implying a significant level of immunocompetence in the neonatal period.

In conclusion, we describe a rodent model of perinatal allogeneic tolerance induction. Using fetal liver cells and neonatal skin grafts instead of adult splenocytes as tolerogens, we achieved a higher frequency of tolerance using a markedly lower cell inoculum than previously described and incurred significantly fewer abortions. Significant levels of splenocyte and bone marrow chimerism and a depressed precursor CTL frequency were noted in tolerant recipients. These findings are consistent with the hypothesis that the fetal liver contains HSCs capable of engraftment in utero. We are currently working to identify the specific engrafting HSC subsets in fetal liver tissue. This model of tolerance induction will be used to determine when HSC engraftment occurs after fetal inoculation, whether multilineage chimerism develops after HSC engraftment, and the optimal timing and dose for HSC inoculation. This information should be useful in developing techniques used for large animal models of in utero allogeneic and xenogeneic tolerance induction and, ideally, in the clinical application of this modality.

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